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Assessment of Chemical Skin-Sensitizing Potency by an *In Vitro* Assay Based on Human Dendritic Cells

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The skin-sensitizing potential of chemicals is an important concern for public health and thus a significant end point in the hazard identification process. To determine skin-sensitizing capacity, large research efforts focus on the development of assays, which do not require animals. As such, an *in vitro* test has previously been developed based on the differential expression of *CREM* and *CCR2* transcripts in $CD34^+$ progenitor-derived dendritic cells (CD34-DC), which allows to classify chemicals as skin (non-)sensitizing. However, skin sensitization is not an all-or-none phenomenon, and up to now, the assessment of relative potency can only be derived using the *in vivo* local lymph node assay (LLNA). In our study, we analyzed the feasibility to predict the sensitizing potency, i.e., the LLNA EC3 values, of 15 skin sensitizers using *in vitro* data from the CD34-DC-based assay. Hereto, we extended the *in vitro*–generated gene expression data set by an additional source of information, the concentration of the compound that causes 20% cell damage (IC20) in CD34-DC. We statistically confirmed that this IC20 is linearly independent from the gene expression changes but that it does correlate with LLNA EC3 values. In a further analysis, we applied a robust linear regression with both IC20 and expression changes of *CREM* and *CCR2* as explanatory variables. For 13 out of 15 compounds, a high linear correlation was established between the *in vitro* model and the LLNA EC3 values over a range of four orders of magnitude, i.e., from weak to extreme sensitizers.

Key Words: **skin-sensitizing potency; VITOSENS; LLNA.**

Skin sensitization results from a series of immunological events following skin contact with low–molecular weight (MW) substances. A number of characteristics determine whether a chemical can function as a contact sensitizer, including the ability to penetrate the epidermis, react with protein to form haptens, and further recognition of this complex by immune cells. Up to now, the appraisal of the skin-sensitizing capacity of chemicals has been evaluated by *in vivo* animal tests (OECD, 2002). Because of the REACH legislation, which stands in Europe since 1 June 2007, over 1,800,000 animals are estimated to have to be sacrificed for determining whether or not a chemical is a skin sensitizer (Rovida and Hartung, 2009). Because of the introduction of such novel regulations and the growing public concern regarding animal testing, the development of an *in vitro* test to identify skin-sensitizing chemicals has become top priority. VITOSENS is one of such newly developed *in vitro* assays that models the immune recognition of chemical allergens in dendritic cells (DCs), which are key immune cells involved in skin sensitization. Microarray analyses revealed that in $CD34^+$ progenitor-derived DC (CD34-DC), the expression profiles of cyclic adenosine monophosphate-responsive element modulator (*CREM*) and monocyte chemotactic protein-1 receptor (*CCR2*) displayed highest discriminating potential between chemical skin sensitizers and nonsensitizers after 6 h of exposure. An initial study with 21 chemicals resulted in a predictive power with a concordance of 89%, a specificity of 97%, and a sensitivity of 82% (Hooyberghs *et al.*, 2008).

In the realm of the above-mentioned chemical regulation, current risk management measures are mostly based on the classification of chemicals as either sensitizers or nonsensitizers. However, contact allergens vary substantially with regard to their relative potency. Skin-sensitizing potency can be defined as the relative ability of a chemical to induce skin sensitization, which is determined by the amount of chemical per unit area required for the acquisition of skin sensitization in a previously naïve individual (van Loveren *et al.*, 2008). For chemical safety assessment, it is of utmost importance to evaluate dose-effect relationships and to classify chemicals according to the strength of their response. Up till now, the local lymph node assay (LLNA) is the only validated method that has provided data that are suitable for comparing the relative potencies of contact allergens. Basically, substances

TABLE 1
Chemical Data Set for Potency Classification

Chemical	CAS N°	EC3 (%)	MW	$LogK_{o/w}$	IC20 (μg/ml)
Oxazolone (Oxa)	15646-46-5	0.003	217.22	1.51	5×10^1
Dinitrofluorobenzene (DNFB)	70-34-8	0.030	186.1	1.83	2×10^0
Bandrowski's Base (BB)	20048-27-5	0.030	318.38	No CAS match	1×10^1
Dinitrochlorobenzene (DNCB)	97-00-7	0.060	202.55	2.27	4×10^0
Dihydroquinone (DHQ)	123-31-9	0.110	110.11	1.03	6×10^0
Methyldibromoglutaronitrile (MdBG)	35691-65-7	0.900	265.93	1.63	2×10^1
2-mercaptobenzothiazole(2-MBT)	149-30-4	1.700	167.24	2.86	7×10^1
Dinitrobenzenesulfonic acid (DNBS)	885-62-1	2.000	270.15	−3.32	6×10^1
Cinnamaldehyde (CA)	104-55-2	3.000	132.16	1.82	1×10^1
Nickel sulfate (Nickel)	10101-97-0	4.800	262.85	No CAS match	2×10^2
Tetramethylthiuram disulfide (TMTD)	137-26-8	5.200	240.43	1.7	2×10^{-2}
Eugenol (Eug)	97-53-0	13.000	164.2	2.73	6×10^1
Citral	5392-40-5	13.000	152.24	3.45	1×10^2
Cinnamic Alcohol (CiAlc)	104-54-1	21.000	132.16	1.84	1×10^4
Geraniol	106-24-1	26.000	154.25	3.47	1×10^3

Note. Chemicals were selected for and ranked by their sensitizing capacity as was identified by their threefold stimulation of lymph node proliferation (EC3) in the LLNA (NIH Publication N° 99-4494 on the LLNA, 1999) (Gerberick *et al.* 2005). The extreme sensitizing chemicals are listed on top (EC3 > 0.1%), followed by the strong (0.1% ≤ EC3 < 1%), moderate (1% ≤ EC3 < 10%), and weak (10% ≤ EC3 ≤ 100%) sensitizers. Physicochemical properties such as MW and estimated octanol-water partition ($logK_{o/w}$) coefficients were derived from the Episuite 4.0 software (Environmental Protection Agency). ''No CAS match'' indicates that no results could be retrieved for this chemical in the software. The mean concentration needed to induce 20% cytotoxicity in CD34-DC (IC20) was derived from experiments on three independent donor samples.

are identified as skin sensitizers in this assay if, at any test concentration, they induce a threefold increase of lymph node cell proliferation in mice compared with the vehicle control, expressed as effective concentration (EC)3 (%). Thus, the intrinsic sensitizing potency of a chemical is defined as a function of the concentration required to elicit a threshold positive response, a stimulation index equal to 3. For this reason, the lower the EC3 value, the greater the relative skin-sensitizing potency of the chemical (Gerberick *et al.*, 2005). Contact allergens that vary 10-fold in EC3 value are then assigned to one of these four subcategories identified with the descriptors "extreme" (EC3 < 0.1%), "strong" (0.1% ≤ EC3 < 1%), "moderate" (1% ≤ EC3 < 10%), and "weak" (10% ≤ EC3 ≤ 100%) (Kimber *et al.*, 2003).

Consequently, a new intriguing question arose: Which critical events are triggered during the induction of skin sensitization that correlates quantitatively with potency? Identification of such events would aid in more accurate predictions of the skin-sensitizing potency of a chemical. DC activation upon allergen recognition is one such possibility, which is the underlying immune reaction event of the VITOSENS assay. Although this activation is often considered as a readout for hazard identification and not risk (Kimber *et al.*, 2009), Gildea *et al.* (2006) observed measurable differences in gene expression changes in DC depending on the potency class of the inducer when applied at a dose that induced 20% cell death. Other evidence that rewards a potency-dependent response in DC is the supposition that chemicals that are stronger sensitizers are also more capable of producing danger signals (Basketter *et al.*, 2007). This coincides with Matzinger's "danger hypothesis," which states that besides allergen recognition, also direct damage of DC or other cells must occur to achieve DC activation (Gallucci and Matzinger, 2001).

To evaluate the prospects of the VITOSENS assay toward potency classification, we explored whether the differential expression of the biomarker genes displays opportunities for categorization of relative sensitization potency. To this end, CD34-DC were exposed to 15 chemicals identified by LLNA studies as weak, moderate, strong, and extreme sensitizers and their *in vitro*–generated responses were correlated with *in vivo* obtained EC3 values.

MATERIALS AND METHODS

Chemical compounds. The selection of the chemical compounds was based on of a number of criteria. First of all, the compound had to be recognized as a sensitizer by the LLNA and VITOSENS assays. To correlate the *in vitro* data to relative *in vivo* skin-sensitizing potency, LLNA EC3 values on each compound were required (National Institutes of Environmental Health Sciences, 1999) (Gerberick *et al.*, 2005). Furthermore, the set of chemicals was chosen to cover the whole range from weak to extreme sensitizing compounds. The chemicals were selected for their physicochemical parameters to favor skin penetration (MW and $logK_{o/w}$ values) (Smith Pease *et al.*, 2003). This resulted in a set of 15 chemicals shown in Table 1. All chemicals were purchased at highest possible purity from Sigma-Aldrich (St Louis), except for Bandrowski's Base (Apollo Scientific, Cheshire, UK).

Cell culture. $CD34^+$ cell isolation and culture procedures have been described before (Schoeters *et al.*, 2007). Briefly, human cord blood samples

were collected from the umbilical blood vessels of placentas of normal full-term infants. Informed consent was given by the mothers, and the study was approved by the ethical commission of the Heilig Hart Hospital at Mol, Belgium, and the St Dimpna Hospital in Geel, Belgium. Mononuclear cells were separated from the cord blood by density gradient centrifugation, and subsequently, $CD34^+$ progenitor cells were extracted by positive immunomagnetic selection. These cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen, San Diego) in the presence of granulocyte/macrophage colony stimulating factor (Gentaur, Brussels, Belgium), stem cell factor (Biosource, Nivelles, Belgium), tumor necrosis factor-α (Roche Applied Science, Upper Bavaria, Germany), and interleukin 4 (Biosource) to induce proliferation and differentiation toward immature CD34-DC according to the method described by Lardon *et al.* (1997). After differentiation, CD34-DC were assessed for phenotypical properties of myeloid DC as was described before (De Smedt *et al.*, 2005; Nelissen *et al.*, 2009). Flow cytometry data are provided as supplemental material (Supplementary figure 1).

Chemical exposure. At the end of the 12-day culture period, the viability of the cells was assessed using propidium iodide staining and immature DC (4×10^6 cells/4 ml/well) from the same donor were exposed to the 15 chemicals in 6-well plates. As was previously published, the predictive capacity of the VITOSENS assay was assessed by a cross-validation based on the gene expression at the time points 6, 11, and 24 h. Later, we repeated the cross-validation using each of the time points separately. The predictive capacity of the model based solely on the 6-h data was equal to that of the original model. For this reason and for practical considerations, the 6-h exposure and not the 11 and 24 h was retained in the final setup of the VITOSENS assay (Hooyberghs *et al.*, 2008). The concentrations of the test compounds used in exposure experiments yielded 20% cell growth inhibition or cell death as compared with solvent-treated cells (20% inhibitory concentration, IC20) at 24 h, as was previously determined on at least three biological donor samples by propidium iodide staining, alamarBlue, or WST-1 cytotoxicity assays. IC20 values are listed in Table 1. For each chemical and its corresponding solvent, DC of at least three independent donors were exposed. Dihydroquinone, dinitrobenzene sulfonic acid (DNBS), and nickel sulfate were dissolved in cell culture medium, whereas the other chemicals were prepared in a 100% dimethylsulfoxide (DMSO) stock solution and then further diluted in medium with a final 0.05% (vol/vol) DMSO concentration. After 6 h of exposure, the cell suspensions were collected, supernatants were removed by centrifugation, and the cell pellets lysed in RLT Buffer (Qiagen, Hilden, The Netherlands) containing 1% (vol/vol) 2-mercaptoethanol (Sigma-Aldrich).

Real-time RT-qPCR. Expression changes of the VITOSENS biomarker genes with highest discriminating potential (*CCR2* and *CREM*) were analyzed (Hooyberghs *et al.*, 2008). Total RNA was extracted from the cell lysates using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and quality was visually inspected for absence of degradation products using agarose gel electrophoresis. For the complementary DNA (cDNA) synthesis, 1 μg of total RNA was used up to a total volume of 25.2 μl (diluted with RNase free water). A mix of 4% (vol/vol) $MgCl_2$ (125mM; Invitrogen), 10% (vol/vol) PCR Buffer (10×; Applied Biosystems, Lennik, Belgium), 10% (vol/vol) dNTPs (40mM; Invitrogen), 5% (vol/vol) Random Hexamers (50μM), 5% (vol/vol) RNase Inhibitor (20 U/μl), and 5% (vol/vol) MuLV Reverse Transcriptase (50 U/μl, all from Applied Biosystems) was added to each RNA sample. A protocol for cDNA synthesis was run on all samples (1 h at 42°C, 5 min at 95°C, and then put on hold at 4°C). After cDNA synthesis, the samples were diluted with Baxter water (Viaflo aqua ad injectabilia AUE0304) up to a volume of 250 μl. Real-time reverse transcriptase (RT)-qPCR reactions were then performed in 20 μl volume containing 20 ng cDNA, 12.5% (vol/vol) 10× PCR Buffer, 10% (vol/vol) dNTP (10mM), 7.5% (vol/vol) $MgCl_2$ (50mM, all from Invitrogen), 2.5% (vol/vol) of each primer (25mM; Eurogentec S.A., Seraing, Belgium), 0.625% (vol/vol) SYBR Green I nucleic acid gel strain (500×; Molecular Probes, Leiden, The Netherlands), 1.25% (vol/vol) fluorescein (100nM; Eurogentec s.a., Seraing, Belgium), and 0.625% (vol/vol) Platinum Taq polymerase (5 U/μl; Invitrogen) in Baxter water. Samples were denatured at 95°C during 5 min. Subsequently, 40 PCR cycles were run consisting of 1 min at 95°C for denaturation, 1 min at the appropriate annealing temperature, and 1 min at 72°C for elongation. RT-qPCR reactions were run in triplicate for each sample on a BioRad iCycler (BioRad, Nazareth, Belgium). For each primer pair analyzed, a nontemplate control was run in parallel. After amplification, a threshold was set for each primer and Cq values were calculated for all samples. Primers were designed using Primer Express Software v3.0 from Applied Biosystems and thoroughly tested (primer sequences are available upon request). Gene expression changes were analyzed using the Biogazelle qBasePlus software (www.qbaseplus.com; Biogazelle, Ghent, Belgium) (Hellemans *et al.*, 2007). The results were analyzed with the ΔΔCt method corrected for gene-specific efficiencies. Gene expression changes were determined as fold changes (FC): ratios of gene expression levels of exposed samples over corresponding solvent control samples. For each sample, transcripts of five reference genes were measured (*GAPDH*, *HPRT*, *SDHA*, *RPLI3A*, and *YWHAZ*), and their stability was assessed as described by Vandesompele *et al.* (2002). To achieve accurate FC, it was set as a criterion that the normalization should be performed to a set of at least three stable reference genes.

Statistical data analysis. All statistical analyses were performed using Matlab2008b software (The MathWorks, Inc., Natick). For each evaluation, the experimental data (EC3, IC20, and gene expression FC) were logarithmically transformed in order to have distributions that are closer to Gaussian ones. Moreover, in the linear regression analyses, the use of logarithmic variables is needed to have an approximate homoscedastic variance. If the regression was performed without logarithmic transformation, the resulting correlation would be artificially high because it would be dominated by a few points in the high value range of the variables. In such a case, the regression would overfit these data points, whereas the rest of the data would all cluster close to the origin.

Ordinary least squares linear regression analyses. An ordinary least squares linear regression analysis was used to measure the correlation between both the EC3 values (%) and the linear fit of the FC of *CREM* and *CCR2* and between EC3 and IC20 (μg/ml) for the chemical sensitizers. Per chemical, the mean log FC or log IC20 of three independent experiments was considered. Both the Pearson and the Spearman rank correlation coefficients were calculated. The correlation between the IC20 and the FC of *CREM* and *CCR2* was also evaluated and was quantified by the variance inflation factor (VIF) (see below).

Variance inflation factor. The VIF is a measure to assess the multicollinearity of the explanatory (or the independent) variables in an ordinary least squares regression analysis. It was used to avoid the inclusion of explanatory variables that are highly correlated because this would lead to unstable estimates of the regression coefficients. The explanatory variables that were considered for this analysis are the IC20 and the FC of *CREM* and *CCR2*.

The VIF for each explanatory variable X_i is defined as $\mathrm{VIF}_i = \frac{1}{1-R_i^2}$, where R_i is the correlation coefficient of an ordinary least square regression with X_i as a function of the other variable X_j. When X_i cannot be predicted by the other X_j, the R_i^2 will be close to 0 and the VIF close to 1. A higher VIF indicates a higher multicollinearity, and a common rule of thumb is that multicollinearity is high when VIF > 5.

Cook's distance and leverage. Cook's distance is an estimate of the influence of a data point in least squares regression. It measures the effect of deleting a given observation on the residuals of all other observations, a higher distance indicates a more influential point.

Leverage points are those observations made at outlying values of the independent explanatory variables, such that the lack of neighboring observations means that the fitted regression model will pass close to that particular observation. If a point has large leverage, then the slope of the regression line follows more closely the slope of the line between that point and the mean point.

M-estimation robust multiple linear regression. Standard linear regression models are based on certain assumptions, such as a Gaussian distribution of errors in the observed responses. If the distribution of errors is

heavy tailed or prone to outliers, model assumptions are invalid and parameter estimates become unreliable. A possible alternative is the use of robust regression models, which are less sensitive to outliers. In the current paper, M-estimation robust multiple linear regression is implemented, which operates by assigning a weight to each data point. Weighting is done by the iteratively reweighted least squares method. In the first iteration, each point is assigned equal weight and model coefficients are estimated using ordinary least squares. At subsequent iterations, weights are recomputed so that points farther from model predictions in the previous iteration are given lower weight. Model coefficients are then recomputed using weighted least squares. The process continues until the values of the coefficient estimates converge within a specified tolerance. Technically, the weights were assigned by the bisquare objective function with a biweight tuning constant of 3.

RESULTS

In the current paper, we analyzed the feasibility to apply data obtained by the *in vitro* VITOSENS assay in a fit to the *in vivo* derived potency of skin sensitizers, determined by the EC3 value of the LLNA. For this research, only sensitizers were retained, which implies that in an operational setup, a compound is only allowed to enter the current potency evaluation if it was previously classified as a sensitizer by the VITOSENS assay.

To this end, RNA samples were generated from CD34-DC that were exposed for 6 h to the IC20 concentration of 15 sensitizing chemicals, and gene expression FC of the markers *CREM* and *CCR2* as compared with solvent-treated cells were obtained using real-time RT-qPCR. Per compound, the gene expression measurements were independently performed on DC from at least three donors. The resulting mean logarithmic FC of *CREM* and *CCR2* are presented as supplemental data (Supplementary figures 2 and 3). In a first statistical analysis approach, the data on gene expression changes of *CREM* and *CCR2* were compared with the LLNA EC3 values. Figure 1 presents the results of an ordinary least squares linear regression analysis between the chemical-induced FC in CD34-DC and the EC3 value. For visual simplicity, only the mean value of the modeled potency per compound is plotted. Although the chemicals oxazolone (Oxa), dinitrochlorobenzene (DNCB), and cinnamic alcohol (CiAlc) distort the relation, an interdependence between both variables is clearly observed. The Spearman rank correlation coefficients and Pearson correlation coefficients (R) are 0.26 and 0.13, respectively.

The presence of outlying chemicals may be brought on by the deficit of explanatory variables. To this end, we extended the gene expression data set of the 15 sensitizers by an additional source of *in vitro* information, the compound's ability to induce damage to CD34-DC. As an (inverse) measure of cell damage potency, we used the chemical concentration that induces 20% cell death or cell growth inhibition (IC20). There are reasons to assume that the IC20 and FC in our assay are independent to a certain degree. First, in our assay, exposure for gene expression measurement occurred at a previously determined chemical-specific IC20 level; hence, in theory, all the exposed cultures experience a level of damage,

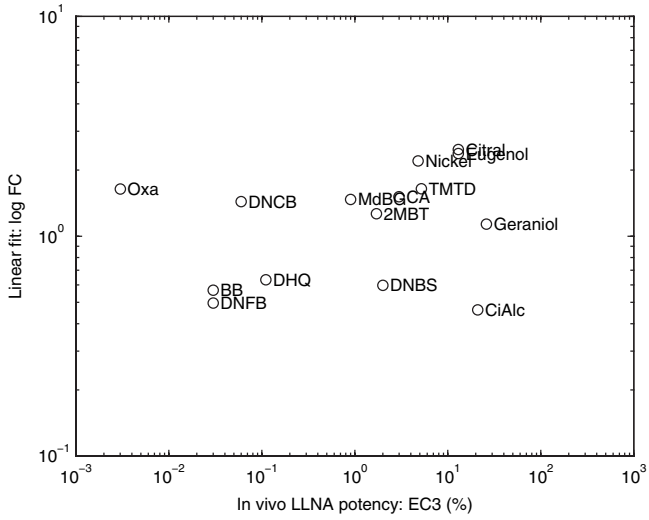

FIG. 1. Scatter plot of the linear fit results of the *in vitro* FC of *CREM* and *CCR2* versus the *in vivo* EC3 per compound. Mean FC values of three independent experiments are used per chemical. Spearman rank correlation coefficient = 0.26 and Pearson correlation coefficient = 0.13.

which is equal and independent of the applied compound. Second, it has been postulated before that the danger effect a compound induces because of damage is strongly related to the exposure concentration of an allergen, whereas the antigenic properties are to a lesser extent related to concentration. Therefore, we assume that because we created an equal level of danger for all chemicals in this setup, the compound-specific response of DC, here the FC of *CREM* and *CCR2*, is dependent on the compound-specific antigenic signal and not on the compound-specific danger-inducing IC20. One way to statistically assess this independence is by evaluating the multicollinearity through the VIF of the IC20 versus the FC of *CREM* and *CCR2*. The resulting VIF = 1.1 showed that the IC20 is not linearly related to the FC, indicating that it potentially possessed additional information worthwhile to include in a model for potency prediction. Applying an ordinary least squares linear regression analysis, we observed a moderate correlation between CD34-DC-derived IC20 and LLNA EC3 values for all 15 chemical sensitizers (Figure 2), involving Spearman rank correlation coefficients and Pearson correlation coefficients (R) of 0.60 and 0.73, respectively. A trend toward a strong correlation is apparent, again with outliers as, e.g., tetramethylthiuram disulfide (TMTD).

The next step would be to perform an ordinary least squares regression analysis with both the IC20 and the FC of *CREM* and *CCR2* as explanatory variables for the dependent variable EC3. However, ordinary least squares regression is known to rely heavily on assumptions, which are often not met in practice and perform poorly in the presence of outliers, either in the explanatory variables or in the dependent variable. Therefore, we first identified the impact of the data on the regression analysis by calculating the leverage and Cook's

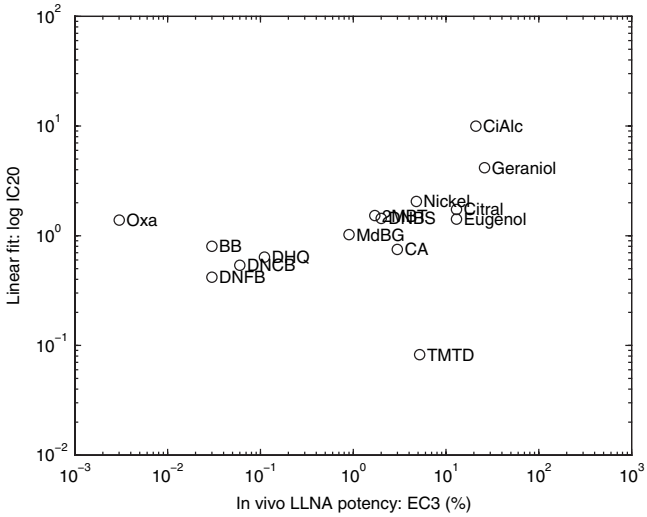

FIG. 2. Scatter plot of the linear fit results of the *in vitro* IC20 versus the *in vivo* EC3 per compound. Spearman rank correlation coefficient = 0.60 and Pearson correlation coefficient = 0.73.

distance. Figure 3 shows the result for the mean over the three experimental replicates. A high Cook's distance is an indicator for high influence of a point on the regression parameters, and in Figure 3a, the compounds Oxa and TMTD are identified as such. In Figure 3b, the leverage per chemical is shown, which is a measure for the outlying character of points in the space of the explanatory variables; the dependent variable is not considered in this measure. There are no pronounced candidates in this sense, indicating the absence of compounds with outlying IC20 or gene expression FC. TMTD and CiAlc are on the high end, which is a consequence of their respective extremely low and high IC20 values as can be seen in Figure 2.

Having observed a few outliers, it is advantageous to perform a robust regression over ordinary least squares regression. In the current study, an M-estimation robust linear regression method was chosen, which iteratively reweights data points giving less weight to outlying data, thereby significantly improving the linear model for the remaining data. The result of the robust linear regression with both the IC20 and the gene expressions as input is shown in Figure 4, the inset explicitly presents all replicate measurements, whereas the main pane depicts the corresponding mean result per compound. All replicate data points of Oxa and TMTD received a final zero weight in the robust model and were marked as outliers. The remaining compounds closely scattered around the identity diagonal. The Spearman rank correlation coefficients and Pearson correlation coefficients are, respectively, 0.91 and 0.79. The contribution of each variable was assessed by its respective significance level; the IC20 had the largest impact on the strong correlation estimates ($p < 0.001$), followed by the FC of *CREM* ($p = 0.050$) and *CCR2* ($p = 0.166$). In conclusion, *in vivo* EC3 values of 13 out of 15 compounds proved a strong linear correlation with IC20 values and gene expression FC of *CREM* and *CCR2* in *in vitro*–cultured CD34-DC. The correlation is valid over four orders of magnitude in EC3; this is over the entire range of the LLNA going from weak to extreme sensitizers. Note that the residuals, i.e., the

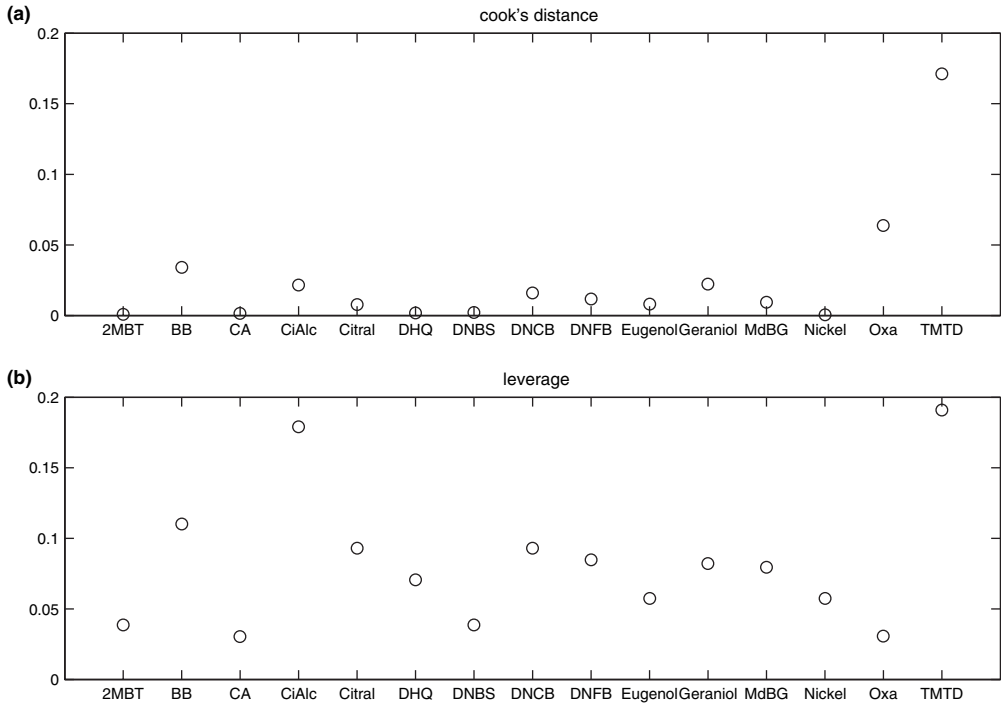

FIG. 3. The Cook's distance (a) and leverage (b) per compound. Mean values derived from at least three different donors are shown per chemical.

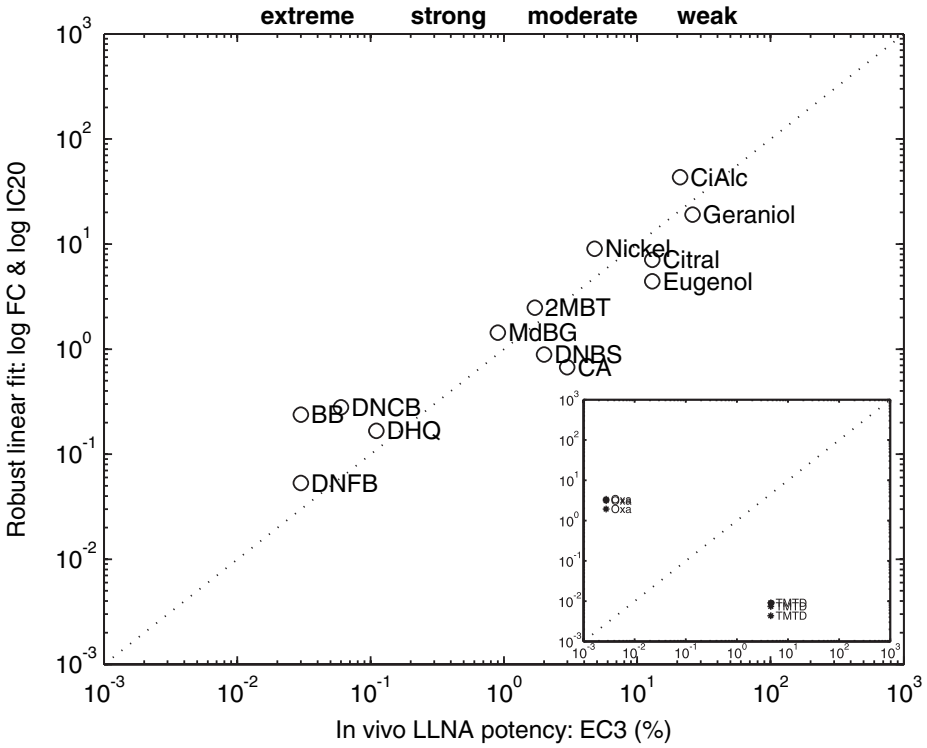

FIG. 4. Scatter plot of robust linear fit results from *in vitro* data (IC20 and FC) versus *in vivo* EC3. The dashed line is the identity diagonal, added as a visual guide. Per compound, the mean result of at least three replicate measurements is shown. After removal of the outlying compounds, Spearman rank correlation coefficient = 0.91 and Pearson correlation coefficient = 0.79. The four subcategories for LLNA potency classification are indicated on top of the graph. The inset shows the individual results of each replicate measurement for all compounds. Data points that received zero weight (outliers) are labeled by the name of the exposure compound.

vertical deviations from the diagonal, are more or less equal over the entire data range, which is a clear indication that the use of logarithmic scales for the variables was needed (see Statistical data analysis in "Materials and Methods" section). Without the logarithmic transformation, the resulting correlation would be higher, but the residuals would be dependent on potency class, which is unwanted.

DISCUSSION

Using data obtained by the *in vitro* VITOSENS assay, we aimed to establish a measure for skin-sensitizing potency in correspondence with the EC3 values obtained by the LLNA. This latter animal-based assay seeks to identify contact allergens as a function of events induced during the acquisition of skin sensitization, more specifically, lymphocyte proliferative responses induced in the regional lymph nodes of mice exposed topically to test chemicals.

Earlier reports suggested that genes expressed in peripheral blood monocyte–derived DC might also be capable of classifying allergens into specific potency categories based on relative differences in gene expression (Gildea *et al.*, 2006). This observation prompted us to investigate the feasibility of assessing the potency of 15 skin-sensitizing chemicals, which were identified as such by the VITOSENS assay. As a first test in the setup, chemicals could be grouped as sensitizers or not based upon the gene expression changes of *CREM* and *CCR2* (Hooyberghs *et al.*, 2008). When in a next step, compounds needed to be ranked according to their sensitizing potency, these FC yielded insufficient information. Therefore, we extended the gene expression data set by another *in vitro*–generated variable, the concentration of the test chemical that yielded 20% cell damage (referred to as IC20). This inclusion is founded upon the danger hypothesis by Matzinger, who described direct damage to DC as a danger signal that might aid in the induction of maturation (Gallucci and Matzinger, 2001). *In vitro*, this referred danger signal may be translated as the mild cell cytotoxicity needed to activate DC (Hulette *et al.*, 2005). ANOVA inflation factors revealed that both IC20 and gene expression changes are linearly independent from one another and therefore could be used as explanatory variables in a linear regression analysis. This might not be surprising for two reasons. First, in our setup, all exposures for gene expression measurement occurred at an IC20, which consequently creates an equal

level of cellular damage and this damage level is therefore independent of the applied compound. Second, it has been postulated that *in vivo*, the concentration of a hapten may be related to its danger signal but may have less to do with antigen signals (McFadden and Basketter, 2000). Therefore, we assume that with an equal level of damage or danger, the compound-specific response of DC (the FC of *CREM* and *CCR2*) is rather dependent on the compound-specific antigen signal and not on the compound-specific IC20. The relevance of including IC20 as an explanatory variable was experimentally demonstrated by a clear trend toward the chemical with lower damaging dose also being more sensitizing.

Because ordinary least squares estimates are highly susceptible to and biased by outliers, we applied a more robust approach, i.e., M-estimation robust multiple linear regression analysis. This analysis clearly confirmed the deviate character of Oxa and TMTD, which also became apparent by applying a Cook's distance analysis. All other data were correlated without accounting any weight to the former compounds. For the remaining chemical sensitizers, a potency value was modeled that closely fitted the *in vivo* EC3 data, and this over the entire range from weak to extremely sensitizing chemicals. This observation points to the feasibility to classify sensitizers further into several potency classes based on our *in vitro* data.

The identification of TMTD and Oxa as chemical sensitizers with distorting impact on the regression fit might indicate some limitations of the proposed model of skin-sensitizing potency. TMTD was identified as a compound with an extremely low IC20 value (0.024 μg/ml) in CD34-DC and was therefore attributed a higher potency in our setup than what was determined *in vivo* by the LLNA (Gerberick *et al*., 2005). This finding might be an indication that the model presented here is not suitable for classifying chemicals with extreme IC20 values. Oxa, on the other hand, did not exert cytotoxicity at extreme concentrations but nonetheless appeared to behave as an outlier in the VITOSENS potency classification. No data could be retrieved on the skin-sensitizing potency in humans, whereas in the LLNA, Oxa was identified as an extreme sensitizer based on its EC3 stimulation (Loveless *et al*., 1996). In our setup, however, Oxa showed up merely as a moderate sensitizer. Besides interspecies differences, a possible explanation for this discrepancy is the fact that Oxa is a compound with poor water solubility, which may mask it's *in vivo* effects in a cell culture environment. A similar phenomenon may occur for DNCB and DNBS, the latter being the water-soluble analogue of the former (Ryan *et al*., 2004). DNBS has a negative $\log K_{o/w}$ value and therefore poor lipid solubility. This is probably the cause of the underestimation of the potency difference between the two compounds in our assay. Nonetheless, addition of the octanol-water partition coefficient ($\log K_{o/w}$) as an explanatory variable appeared not sufficient (data not shown) to improve the fitted model. Hence, besides expansion of the chemical data set, another possible step to further optimize the current *in vitro* potency classification is the inclusion of additional explanatory variables that impact on the acquisition of skin sensitization. Integration of parameters like epidermal disposition, peptide reactivity, or T-cell proliferation may contribute in approximating this biological complexity (Jowsey *et al*., 2006) and may therefore aid in improving relative potency estimates.

A final observation is the close fit between VITOSENS and LLNA skin-sensitizing potency of chemicals that require metabolic activation to become an active sensitizer, so-called prohaptens (e.g., eugenol, geraniol, and CiAlc). This suggests that CD34-DC possess metabolic capacities.

In conclusion, we propose that besides the LLNA, an *in vitro* assay based on the activation of primary DC may provide valuable information in determining the relative potency of chemical skin sensitizers. When a compound is being identified as a skin sensitizer by the VITOSENS assay, further evaluation of the obtained data quantitatively fits its sensitizing capacity with strong correlation to the LLNA EC3 values. This holds for the entire range of chemicals tested in the LLNA going from weak, even including prohaptens, to extreme sensitizers. Therefore, we believe that the VITOSENS assay offers a relevant source of *in vitro* information for future identification of safe-to-use exposure thresholds of potential skin sensitizers and that the underlying event of DC activation is not an all-or-none phenomenon. Further compound testing and characterization of the applicability domains, as well as limitations of this model, are required to allow for *in vitro* potency classification. Effective integration of VITOSENS in a test battery alongside other test systems that reflect different steps of the sensitization cascade may be a possible solution for approximating the biological complexity of the process.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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